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(54) Pharmaceutical preparation containing human growth hormone

(57) A stable pharmaceutical preparation containing a human growth hormone having a molecular weight of about 20,000, or a derivative thereof, can be formulated by adding a water soluble heterocyclic compound, such as creatinine, a salt of acetyltryptophane and nicotinamide, to prevent the insolubilization of the human growth hormone or the derivative thereof in an aqueous solution.

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Description

The present invention relates to pharmaceutical preparations containing a human growth hormone having a molecular weight of about 20,000 (hereinafter referred to as 20k hGH), more specifically to lyophilized preparations prepared from a solution containing 20k hGH, which have excellent storage stability and do not produce any foreign or insoluble matter when reconstituted, and to processes for the production thereof.

There are two known types of human growth hormone: one having a molecular weight of about 22,000 (22k hGH) and the other having a molecular weight of about 20,000 (20k hGH). The 22k hGH is produced by means of recombinant DNA technology and is used for treatment of pituitary dwarfism in the field of pediatrics. The 20k hGH has never been produced on an industrial scale and it has never been used for medical treatment.

20k hGH is a single-chain polypeptide having a molecular weight of about 20,000 and has an isoelectric point of about 5.5. Thus, it is stable in an aqueous solution at a neutral pH but unstable at acid and alkaline pH ranges. The solubility of 20k hGH in aqueous solutions at weak acid to weak alkaline pH ranges is less than about 1 mg/ml and insoluble matter is produced upon thawing out the frozen solution. Thus, 20k hGH can be considered to be a protein with very low solubility. Furthermore, in aqueous solutions at weak acid to weak alkaline pH ranges, 20k hGH easily dimerizes. It has been reported that 20k hGH derived from the human pituitary gland often co-dimer-izes or dimerizes with 22k hGH, a human growth hormone having a molecular weight of about 22,000 (Chapman et al., J. Biol. Chem., Vol. 256, 2395-2401, 1981). These facts suggest that the low solubility of 20k hGH is due to a hydrophobic interaction of protein molecules.

In order to improve the solubility of highly hydrophobic proteins, sodium dodecyl sulfate, which is extremely surface active, or denaturing agents such as urea and guanidine hydrochloride, and the like are generally used. However, these agents destroy the protein structure, and the primary functions of the proteins will be lost or weakened. Therefore, the use of these conventional agents are not at all preferable if the proteins are to be used in pharmaceutical preparations.

On the other hand, in one known example of the use of solubility promoters to improve the solubility of proteins, equimolar quantities of histidine and creatinine, having a positive charge, and citric acid, having a negative charge, were added to improve the solubility of a modified form of tissue plasminogen activator (hereinafter referred to as tPA) (US 4,980,165). tPA and modified tPA are proteins which are extremely insoluble at neutral pH ranges but highly soluble at acid pH ranges. In other words, the low solubility of tPA and modified tPA is caused by isoelectric precipitation of the proteins, which this method suppresses by the addition of histidine and creatinine, having a positive charge, and citric acid, having a negative charge.

Prescriptions of pharmaceutical preparations containing a human growth hormone having a molecular weight of about 22,000, which are commercially available today, are shown in Table 1. These preparations are generally administered subcutaneously or intramuscularly.

Further, these 22k hGH preparations primarily contain glycine or mannitol and are stable when stored at 5°C for 1 year.

Table 1:

Prescription of commercially available lyophilized 22k hGH				
Name of product	Additive(s)		Solution reconstitution	
Genotropin (Kabi Pharmacia Sumitomo)	4IU	Glycine:24 mg	Water for injection	1 ml
Norditropin (Nordisk)	4IU	Glycine:24 mg D-Mannitol:2.4 mg	Water for injection	1 ml
Humatrope (Lilly)	4IU	Glycine:1.48 mg D-Mannitol:7.4 mg	Saline	2 ml
Saizen (Serono)	4IU	D-Mannitol:20 mg	Saline	1 ml
Groject (Bio-Tech General)	4IU	D-Mannitol:40 mg	Saline	1 ml

A study by the present inventors showed that stable preparations could not be obtained when lyophilized 20k hGH preparations were produced as above.

If a stable aqueous solution of 20k hGH of sufficient concentration cannot be obtained for use in producing preparations to be administered as described above, then the dosage would have to be increased. This can be extremely inconvenient in the case of 20k hGH preparations for injection.

Several compounding methods are known to stabilize 22k human growth hormones in solution. Reported examples include the addition of arginine and EDTA as stabilizing agents for an aqueous 22k hGH solution (EP -A-639984), the addition of polyhydric alcohols or amino acids in order to control the production of insoluble matter and maintain activity of soluble matter in a 22 khGH solution (EP-A-303746), and the addition of histidine as a stabilizing agent in order to

suppress an increase in related substances in an aqueous 22 khGH solution (EP-A-618807). However, all of these methods were developed to stabilize 22 khGH in a solution, and do not refer to the stabilization of a lyophilized 22k hGH product. Furthermore, nothing is known about stabilization of a lyophilized preparation of 20k hGH.

The present inventors studied the solubility and stability of 20k hGH. As shown in Table 2, the result showed that aside from being of lower molecular weight than 22k hGH, 20k hGH is quite different from 22k hGH in physicochemical properties such as physiological activity, stability and solubility. In particular, the original 20k hGH bulk solution is unstable even after lyophilization. When its lyophilized preparation is stored at a temperature as low as 5°C, the quantities of related substances, such as a deamidated variant in which Asp^{134} in 20k hGH is deamidated to Asp^{134} and a sulfoxide variant in which Met^{14} was converted to ox-Met^{14}, and high molecular weight poymer products, increased over time. The related substances means, for example, a mono-deamidated variant in which Asn^{134} in 20k hGH is deamidated to Asp^{134}, a di-deamidated variant in which besides Asn^{134}, Asn^{137} was also deamidated, and a sulfoxide variant in which Met^{14} was converted to ox-Met^{14}. Furthermore, in handling, for example, pipetting, a solution in which the 20k hGH was dissolved, the protein were readily aggregated to produce insoluble matter. In other words, the stability in an aqueous solution is low. As described hereinafter, even the addition of basic amino acids to a 20k hGH solution does not suppress the production of insoluble matter or related substances. As discussed above, 20k hGH is highly hydrophobic, which may explain why it tends to produce more insoluble matter than 22k hGH.

Table 2:

Difference in physicochemical properties between 20k hGH and 22k hGH		
Physicochemical property	20k hGH	22k hGH
Isoelectric point	pH 5.5 [1]	pH 5.1[1]
Dimer formation	Easily formed[2]	Hardly formed[2]
Hydrophobicity	High[3]	Low[3]
Solubility in water	Low[3]	High[3]
Stability of lyophilized hGH	Low [3]	High[3]
Stability of dissolved hGH	Low[3]	High[3]

1: Endocrine Reviews, Vol. 12, 314-324, 1991.

2: J. Biol. Chem., Vol. 256, 2395-2401, 1981.

3: Data by the present inventors.

As described above, it is very difficult to obtain a solution in which 20k hGH maintains its physiological activity in a soluble and stable form simply, by applying the conventional compounding preparations of 22 khGH. Accordingly, there is a strong need to develop new compounding prescriptions for stable 20k hGH pharmaceutical preparations which retain an appropriate concentration for injection.

Furthermore, since lyophilization of 20k hGH alone cannot prevent the production of related substances and high molecular weight polymer products, there is a need to develop a stable lyophilized pharmaceutical preparations containing 20k hGH, which produce very little related substances or high molecular weight polymer products.

Accordingly, the present invention seeks to provide pharmaceutical preparations which contain 20k hGH and produce very little insoluble matter derived from the 20k hGH when dissolved in water. The present invention further seeks to provide lyophilized preparations containing 20k hGH which suppresse the production of related substances and high molecular weight polymer products after reconstitution.

Additionally, the present invention seeks to provide methods to prevent the insolubilization of 20k hGH in a pharmaceutical preparation containing 20k hGH in order to improve its stability, and a method to suppress the production of related substances and high molecular weight polymer products over time in a lyophilized preparation containing 20k hGH.

The present inventors did extensive studies to achieve the above, that is, to improve the solubility and stability of 20k hGH and to provide a compounding prescription for a stable 20k hGH preparation upon lyophilization. As a result, the present inventors have succeeded in producing a pharmaceutical preparation containing 20k hGH which has excellent solubility and stability.

Furthermore, the present inventors found that when a basic amino acid and a nonionic surfactant are added to a lyophilized preparation containing 20k hGH, the production of related substances of the lyophilized preparation was suppressed to improve stability, such that said lyophilized preparation did not produce any insoluble matter when reconstituted in water.

The present invention comprises pharmaceutical preparations containing a human growth hormone having a molecular weight of about 20,000, or a derivative thereof, and a water soluble heterocyclic compound; methods to prevent insolubilization of a human growth hormone having a molecular weight of about 20,000, or a derivative thereof, thereby

improving the stability of a pharmaceutical preparation containing said human growth hormone or a derivative thereof by adding a water soluble heterocyclic compound; and methods to suppress the production of related substances over time in a lyophilized preparation containing a human growth hormone having a molecular weight of about 20,000, or a derivative thereof, by adding one or two kinds of basic amino acids, or salts thereof, and a nonionic surfactant.

The present inventors found that the solubility of 20k hGH can be improved by adding a water soluble heterocyclic compound, such as creatinine, in a certain concentration to a 20k hGH preparation in which the use of buffer solutions generally used for physiological substances would not provide a sufficient concentration of 20k hGH for use as a pharmaceutical preparation.

Furthermore, it is possible to prevent the production of extremely small amounts of insoluble matter by controlling the pH of an aqueous 20k hGH. It was also found that the stability in solution upon thawing can be improved by adding a nonionic surfactant such as polysorbate 80, and furthermore, stability upon lyophylized preparation and during reconstitution can be improved by adding basic amino acids and mannitol, a sugar alcohol. These findings readily enable the mass production of pharmaceutical preparations containing lyophilized 20k hGH which can be reconstitution to prepare aqueous solutions suitable for injection.

The growth hormone according to the present invention is a human growth hormone having a molecular weight of about 20,000 (20 kHG) and said human growth hormone can be either a natural hormone or one obtained by means of recombinant DNA technology.

Examples of the 20k hGH according to the present invention include those having amino acid sequences shown below, i.e., SEQ ID NO: 1 and SEQ ID NO: 2; however, those in which one or several amino acids in the entire amino acid sequence of SEQ ID NO: 1 or SEQ ID NO: 2 are different are also within the scope of the invention as long as the resultant 20k hGH has retained its physiological characteristics.

SEQ ID NO: 1

```
Phe Pro Thr Ile Pro Leu Ser Arg Leu Phe Asp Asn Ala Met Leu
 1               5                  10                  15

Arg Ala His Arg Leu His Gln Leu Ala Phe Asp Thr Tyr Gln Glu
            20                  25                  30

Phe Asn Pro Gln Thr Ser Leu Cys Phe Ser Glu Ser Ile Pro Thr
        35                  40                  45

Pro Ser Asn Arg Glu Glu Thr Gln Gln Lys Ser Asn Leu Glu Leu
    50                  55                  60

Leu Arg Ile Ser Leu Leu Leu Ile Gln Ser Trp Leu Glu Pro Val
65                  70                  75

Gln Phe Leu Arg Ser Val Phe Ala Asn Ser Leu Val Tyr Gly Ala
            80                  85                  90

Ser Asp Ser Asn Val Tyr Asp Leu Leu Lys Asp Leu Glu Glu Gly
        95                  100                 105

Ile Gln Thr Leu Met Gly Arg Leu Glu Asp Gly Ser Pro Arg Thr
    110                 115                 120

Gly Gln Ile Phe Lys Gln Thr Tyr Ser Lys Phe Asp Thr Asn Ser
125                 130                 135

His Asn Asp Asp Ala Leu Leu Lys Asn Tyr Gly Leu Leu Tyr Cys
            140                 145                 150

Phe Arg Lys Asp Met Asp Lys Val Glu Thr Phe Leu Arg Ile Val
        155                 160                 165

Gln Cys Arg Ser Val Glu Gly Ser Cys Gly Phe
    170                 175
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SEQ ID NO:2

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Phe Pro Thr Ile Pro Leu Ser Arg Leu Phe Asp Asn Ala Ser Leu
 1               5                  10                  15

Arg Ala His Arg Leu His Gln Leu Ala Phe Asp Thr Tyr Gln Glu
            20                  25                  30

Phe Asn Pro Gln Thr Ser Leu Cys Phe Ser Glu Ser Ile Pro Thr
        35                  40                  45

Pro Ser Asn Arg Glu Glu Thr Gln Gln Lys Ser Asn Leu Glu Leu
    50                  55                  60

Leu Arg Ile Ser Leu Leu Leu Ile Gln Ser Trp Leu Glu Pro Val
65                  70                  75

Gln Phe Leu Arg Ser Val Phe Ala Asn Ser Leu Val Tyr Gly Ala
                80                  85                  90

Ser Asp Ser Asn Val Tyr Asp Leu Leu Lys Asp Leu Glu Glu Gly
            95                  100                 105

Ile Gln Thr Leu Met Gly Arg Leu Glu Asp Gly Ser Pro Arg Thr
        110                 115                 120

Gly Gln Ile Phe Lys Gln Thr Tyr Ser Lys Phe Asp Thr Asn Ser
    125                 130                 135

His Asn Asp Asp Ala Leu Leu Lys Asn Tyr Gly Leu Leu Tyr Cys
140                 145                 150

Phe Arg Lys Asp Met Asp Lys Val Glu Thr Phe Leu Arg Ile Val
            155                 160                 165

Gln Cys Arg Ser Val Glu Gly Ser Cys Gly Phe
        170                 175
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The concentration of 20k hGH contained in a pharmaceutical preparation containing 20k hGH according to the present invention is 0.5 to 10.0 mg/ml, preferably 1.0 to 3.0 mg/ml, more preferably 1.5 to 2.5 mg/ml, before lyophilization. Furthermore, effective concentrations of 20k hGH in reconstituted solutions of lyophilized preparations is less than 10 mg/ml.

Pharmaceutical preparations containing 20k hGH of the present invention contain a water soluble heterocyclic compound in order to improve the solubility of 20k hGH in a solution of the preparation, and in a solution of a reconstituted lyophilized preparation. The water soluble heterocyclic compound to be used in the present invention is one or more of the compounds selected from the group consisting of creatinine, a salt of acetyltryptophane such as sodium acetyltryptophane and nicotinamide; however, creatinine is preferable. The concentration of said water soluble heterocyclic compound in a solution of the preparation or in a solution of a reconstituted lyophilized preparation is 0.1% to 10%, preferably 0.3% to 5%.

The pH of a pharmaceutical preparation containing 20k hGH of the present invention is 5 to 8, preferably 6.0 to 7.8, more preferably 6.5 to 7.6.

When pH adjustment is necessary, a simple pH adjusting agent, such as hydrochloric acid, phosphoric acid and

sulfuric acid can be used, or a buffer solution such as tris(hydroxymethyl)aminomethane, phosphoric acid, maleic acid, succinic acid, citric acid, acetic acid, histidine or salts thereof can be used. Phosphoric acid, or a salt thereof, is preferable. The concentration of said buffer solution is 0.1 mM to 100 mM, preferably 5 mM to 50 mM.

The basic amino acids which may be used in the lyophilized preparation containing 20k hGH of the present invention are, for example, lysine, histidine, arginine or salts thereof. The amount of the basic amino acid to be added is 1 to 20 parts by weight to 1 part by weight of 20k hGH, preferably 2 to 15 parts by weight.

Furthermore, lyophilized preparations containing 20k hGH according to the present invention may contain a nonionic surfactant to improve the stability of 20k hGH after reconstitution of said lyophilized preparation. The nonionic surfactants to be used in the present invention are polyoxyethylenepolyoxypropyleneglycol and polysorbate, e.g., polyoxyethylene (160) polyoxypropylene (30) glycol, polysorbate 20 or polysorbate 80. Polysorbate 20 or polysorbate 80, or both are preferable. Polysorbate 80 is more preferable. The concentration of nonionic surfactant in a solution of a reconstituted lyophilized preparation is 0.02% to 1%, preferably 0.02% to 0.2%.

Furthermore, lyophilized preparations containing 20k hGH of the present invention can be compounded with an excipient to improve the appearance of the cake upon lyophilization. An example of the excipient is a sugar alcohol, e.g., mannitol. The concentration of said excipient is 0.1% to 5%, preferably 0.5% to 2%, in a solution of a reconstituted lyophilized preparation.

The pH of pharmaceutical preparations of the present invention can be adjusted after adding a diluted solution of 20k hGH to an aqueous solution supplemented with the abovementioned water soluble heterocyclic compound.

Furthermore, it is preferable to convert the pharmaceutical preparations of the present invention into lyophilized products. There are no restrictions as to methods and conditions for producing the lyophilized preparation. For example, lyophilization can be carried out by adding a diluted 20k hGH solution to an aqueous solution supplemented with a water soluble heterocyclic compound of the abovementioned concentration, and optionally a specified amount of basic amino acid, a nonionic surfactant, and an excipient, if necessary, adjusting the pH, freezing the resulting admixture at -30°C to -80°C, and then drying under reduced pressure by a conventional method.

Furthermore, when pharmaceutical preparations of the present invention are used for injections, the lyophilized preparations may be reconstituted with appropriate water for injections or solutions containing osmotic pressure adjustmenting agents such as sodium chloride or dextrose or sugar alcohols.

The present invention will be explained in more detail by the following examples; however, the present invention is not limited to those examples.

Example 1: Effect of addition of creatinine on solubility of 20k hGH

20k hGH used in Examples 1 to 9 was prepared by means of recombinant DNA technology. More specifically, it was prepared according to the method described in US 5,496,713 using a transformant strain, MT-10765 (deposited with Accession Number FERM BP-5020 at the National Institute of Bioscience and Human-Technology of the Agency of Industrial Science & Technology of the Ministry of International Trade and Industry, Japan according to the Budapest Treaty; Deposition Date; February 28, 1995). Namely, an expression and secretion plasmid carrying the gene encoding 20k hGH was introduced in *Escherichia coli* and the resulting transformant, MT-10765, was cultured in a medium containing polypeptone, yeast extract, glycerol, etc. After completing the culture, the bacterial cells were harvested by centrifugation and the outer membranes of the cells were burst by the osmotic shock method to recover the periplasm fraction only. Isolation and purification of 20k hGH from the periplasm fraction were carried out according to known methods or their variations.

Urea was added to an aqueous solution containing 0.2 mg/ml to 0.4 mg/ml of 20k hGH obtained as above, and the admixture was concentrated to about 8 mg/ml. Portions of the resultant concentrated solution were added to gel filtration columns each equilibrated with a 20 mM sodium phosphate buffer solution (pH 6.5) containing creatinine in concentrations of 0%, 0.3% (27.8 mM), 0.6% (55.5 mM), 1.25% (11.1 mM), 2.5% (221 mM) and 5% (442 mM), respectively. Urea was removed by gel filtration and the purified fractions were eluted by being replaced by 20 mM sodium phosphate buffer containing creatinine at the abovementioned, various concentrations, respectively. The protein concentration of each purified fraction was measured and change in appearance of solution of the fractions was visually evaluated. The effect of the addition of creatinine are shown in Table 3. From the results in Table 3, it was confirmed that creatinine in the solution improved the solubility of 20k hGH, and furthermore, the concentration of 20k hGH in the purified fractions obtained by gel filtration increased with an increase in the creatinine concentration. Evaluation of other heterocyclic compounds to be used in the present invention, i.e., sodium acetyltryptophane and nicotinamide, showed that they have similar effects as creatinine.

Furthermore, similar results were obtained when an experiment was carried out using 20k hGH which was obtained using the transformant MT-10712 (deposited with the Accession Number FERM BP-4361 at the National Institute of Bioscience and Human-Technology Agency of Industrial Science & Technology of the Ministry of International Trade and Industry, Japan according to the Budapest Treaty; Deposition Date: July 12, 1993), by the same procedure as

described above.

These results showed that the addition of creatinine was effective without the addition of an equimolar amount of a negative charged compound. This suggests that the addition of creatinine in affecting the solubility of 20k hGH is not caused by preventing isoelectric precipitation.

Table 3:

Effect of addition of creatinine on solubility of 20k hGH		
Creatinine concentration in solution (%)	Purified fraction from gel filtration	
	appearance of solution	Concentration (mg/ml)
0	+	Not measured
0.3	-	2.9
0.6	-	3.1
1.25	-	4.1
2.5	-	4.5
5.0	-	4.8
Note: +: precipitates produced; -: clear.		

Example 2: Effect of addition of polysorbate 80 on the appearance of solution of 20k hGH aqueous solution after freezing/thawing.

Polysorbate 80 (commercial name: Tween 80) was added individually at a concentration of 0, 0.005, 0.01, 0.02, 0.05, 0.1 and 0.2% to the 20k hGH solution containing 1.25% (111 mM) of creatinine as shown in Table 3 above. The visual change in each 20k hGH solution before freezing and after thawing was observed in a container having four sides made of clear glass under 6,000 luxes of fluorescent lamp. The 20k hGH concentration used was about 2 mg/ml. The effect of the addition of polysorbate 80 is shown in Table 4. Results revealed that the addition of polysorbate 80 at a concentration of more than 0.02% prevented the production of small amounts of insoluble matter of 20k hGH caused by freezing and thawing, and thus proving that the solution remains stable.

Table 4:

Effect of addition of polysorbate 80 on solubility of 20k hGH after freezing/thawing		
Concentration of polysorbate 80 in solution (%)	Change in appearance of solution before freezing and after thawing of 20k hGH aqueous solution	
	Before freezing	After thawing
0	-	++
0.005	-	+
0.01	-	+
0.02	-	-
0.05	-	-
0.1	-	-
0.2	-	-
Note: -: Clear; +:with slightly insoluble matter; ++:with obviously insoluble matter.		

Example 3: Effects of addition of mannitol on appearance of both 20k hGH lyophilized cake and of solution after reconstitution

Mannitol, as an excipient, was added at a concentration of 0, 1.0 and 5.0% to aqueous solutions of 20k hGH which contained polysorbate 80 at a concentration of 0.05% and 0.2% as shown in Table 4 and creatinine at a concentration of 1.25% (111 mM), respectively. The admixtures were dispensed into separate vials, which were cooled from 5°C to -40°C, and then frozen at -40°C for 5 hours. The temperature was then raised from -40°C to -25°C under reduced pressure, after which the vials were dried under reduced pressure at -25°C for another 60 hours. Next, the temperature was increased to 15°C under reduced pressure, and the vials were dried at this temperature for 6 hours. After observing the appearance of the resulting lyophilized cakes, 1 ml of distilled water for injection was added to each cake to observe the appearance of the reconstituted solution under 6,000 luxes of fluorescent lighting. The effect of the addition of

mannitol is shown in Table 5. Results in Table 5 show that the appearance and formability of the lyophilized cakes are improved by the addition of mannitol. Furthermore, it is confirmed that reconstitution is excellent without insoluble matter.

Table 5:

Effects of addition of mannitol on appearance of both lyophilized preparation of 20k hGH and of solution after reconstituion				
Concentrations of solvent composition (%)			Appearance of lyophilized cake	Appearance of solution after reconstitution
Creatinine	Polysorbate 80	Mannitol		
1.25	0	0	Δ	++
1.25	0.05	0	○	-
1.25	0.05	1.0	◎	-
1.25	0.05	5.0	◎	-
1.25	0.2	0	○	-
1.25	0.2	1.0	◎	-
1.25	0.2	5.0	◎	-
Note: -: clear; +: with minor insoluble matter; ++: with clearly visible insoluble matter; Δ: slightly poor; ○: good; ◎: excellent.				

Example 4: Effect of addition of basic amino acids and/or mannitol on production of insoluble matter after reconstitution of 20k hGH lyophilized preparation

Aqueous solutions containing 2 mg/ml of 20k hGH, specified amounts of basic amino acid or hydrochloride thereof, 1.25% (111 mM) creatinine, 2.5% mannitol and 0.05% polysorbate 80, having a pH 7.6 adjusted with sodium dihydrogenphosphate and sodium hydroxide, were prepared. Then, 1 ml portions of the solutions were dispensed into vials, which were cooled from 5°C to -40°C, and then frozen at -40°C for 5 hours. The temperature was then raised from -40°C to -25°C under reduced pressure, after which the vials were dried under reduced pressure at -25°C for another 60 hours. Next, the temperature was increased to 15°C under reduced pressure, and the vials were dried at this temperature for 6 hours. The resulting lyophilized preparations were reconstituted with 1 ml of distilled water for injection and stored at 5°C for 7 days. The state of solution at day 0 and day 7 was observed under 6,000 luxes of fluorescent lamp. Results are shown in Table 6.

Table 6:

Effect of basic amino acids on production of insoluble matters in solution				
Basic amino acid/mannitol			State of solution	
Amino acid	Amount added (mg)	pH	Day 0	Day 7
-	0	6.5	-	+
-	0	7.6	-	-
Arginine hydrochloride	4.2	7.6	-	+
Arginine hydrochloride	6.0	7.6	-	+
Arginine hydrochloride + mannitol	4.2	7.6	-	-
Arginine hydrochloride + mannitol	6.0	7.6	-	-
Histidine	6.0	7.6	-	+
Lysine hydrochloride	6.0	7.6	-	+
Note: -: clear; ±: with very little insoluble matter; +: with little insoluble matter; ++: with clearly visible insoluble matter.				

As shown in Table 6, basic amino acids did not suppress the production of insoluble matter in a 20k hGH solution, and instead had a detrimental effect. These results confirmed that the production of insoluble matter was suppressed by adding mannitol and controlling the pH.

Example 5: Effect of addition of basic amino acids on increasing related substances in solution after reconstitution of 20k hGH lyophilized preparation

An experiment was carried out in the same manner as described in Example 4, except that the pH of an aqueous solution containing 20k hGH was adjusted to 7.6. Related substances (deamidated variants and sulfoxide variants) were qualitatively measured by liquid chromatography on day 0 and day 7. Results are shown in Table 7.

Table 7:

Effect of basic amino acids on production of related substances					
Basic amino acid added		Analogous substances (%)			
Amino acid	Amount added (mg)	pH	Day 0	Day 7	Increase
-	0	7.6	5.1	5.4	0.3
Arginine hydrochloride	4.2	7.6	5.1	5.5	0.4
Arginine hydrochloride	6.0	7.6	5.1	5.5	0.4
Histidine	6.0	7.6	5.2	5.5	0.3
Lysine hydrochloride	6.0	7.6	5.2	5.6	0.4

As shown in Table 7, the addition of basic amino acids had no effect, and neither increased nor decreased the amount of related substances in solution.

Example 6: Effect of addition of arginine and arginine hydrochloride on increase in related substances in 20k hGH lyophilized preparation

Aqueous solutions each containing 2 mg/ml of 20k hGH, specified amounts of arginine or arginine hydrochloride, 1.25% (111 mM) creatinine, 0.05% polysorbate 80, having a pH adjusted to 7.6 with sodium dihydrogenphosphate and sodium hydroxide, were prepared. Then, 1 ml portions of the solutions were dispensed into vials, which were cooled from 5°C to -40°C, and then frozen at -40°C for 5 hours. The temperature was then raised from -40°C to -25°C under reduced pressure, after which the vials were dried under reduced pressure at -25°C for another 60 hours. Next, the temperature was increased to 15°C under reduced pressure, and the vials were dried at this temperature for 6 hours. The resulting lyophilized preparations were stored at 40°C for 2 weeks. Concentrations of related substances were measured at day 0 and 2 weeks later by liquid chromatography. Results are shown in Table 8.

As shown in Table 8, the addition of arginine or arginine hydrochloride suppressed the production of related substances.

Table 8:

Effect of addition of arginine or arginine hydrochloride on production of related substances in lyophilized preparation				
Basic amino acids		Analogous substances (%)		
Amino acid	Amount added (mg)	Day 0	After 2 weeks	Increase
-	0	3.9	10.7	6.8
Arginine hydrochloride	1.05	3.7	7.0	3.3
Arginine hydrochloride	4.20	3.8	5.6	2.2
Arginine hydrochloride	6.00	3.7	5.5	1.8
Arginine hydrochloride	26.7	7.6	8.2	0.6
Arginine	4.96	3.9	7.0	3.1

Example 7: Effect of addition of lysine hydrochloride and histidine on increase in related substances in 20k hGH lyophilized preparation

Aqueous solutions each containing 2 mg/ml of 20k hGH, specified amounts of lysine hydrochloride or histidine, 1.25% creatinine, 0.05% polysorbate 80, having a pH adjusted to 7.6 with sodium dihydrogenphosphate and sodium hydroxide, were prepared. Then, 1 ml portions of the solutions were dispensed into vials, which were cooled from 5°C to -40°C, and then frozen at -40°C for 5 hours. The temperature was then raised from -40°C to -25°C under reduced pressure, after which the vials were dried under reduced pressure at -25°C for another 60 hours. Next, the temperature

was increased to 15°C under reduced pressure, and the vials were dried at this temperature for 6 hours. The resulting lyophilized preparations were stored at 40°C for 2 weeks. Concentrations of related substances were measured at day 0 and 2 weeks later by liquid chromatography. Results are shown in Table 9.

As shown in Table 9, the addition of a basic amino acid and salt thereof, i.e., histidine and lysine hydrochloride affected to suppress the production of related substances in the same manner as the abovementioned arginine and arginine hydrochloride.

Table 9

Effect of addition of basic amino acid on production of related substances in lyophilized preparation				
Basic amino acids	Related substance (%)			
Amino acid	Amount added (%)	Day 0	After 2 weeks	Increase
-	0	3.9	10.7	6.8
Histidine	4.4	3.8	5.8	2.0
Lysine hydrochloride	5.2	3.9	5.6	1.8

Example 8: Effect of addition of basic amino acids on increase in high molecular weight polymer products in 20k hGH lyophilized preparation

Aqueous solutions each containing 2 mg/ml of 20k hGH, specified amounts of basic amino acids, 1.25% creatinine, 0.05% polysorbate 80, having a pH adjusted to 7.6 with sodium dihydrogenphosphate and sodium hydroxide, were prepared. Then, 1 ml portions of the solutions were dispensed into vials, which were cooled from 5°C to -40°C, and then frozen at -40°C for 5 hours. The temperature was then raised from -40°C to -25°C under reduced pressure, after which the vials were dried under reduced pressure at -25°C for another 60 hours. Next, the temperature was increased to 15°C under reduced pressure, and the vials were dried at this temperature for 6 hours. The resulting lyophilized preparations were stored at 40°C for 2 weeks. Concentrations of high molecular weight polymer products were assayed by electrophoresis (SDS-PAGE) at day 0 and 2 weeks later. Namely, the lyophilized preparations in the vials were dissolved with water for injection and heated for 3 minutes with and without a reducing agent (mercaptoethanol) in a boiling water bath. Each sample solution was added (10 μg per well) to a polyacrylamide gradient gel for electrophoresis at constant current with subsequent silver staining. Results are shown in Table 10.

As shown in Table 10, the addition of basic amino acids suppresses the production of high molecular weight polymer products.

Table 10

Effect of addition of basic amino acid on production of high molecular weight polymer products in lyophilized preparation			
Basic amino acids	High molecular weight polymer products (after 2 weeks)		
Amino acid	Amount added (mg)	Unreduced	Reduced
None	0	+++	++
Arginine hydrochloride	2.1	++	+
Arginine hydrochloride	4.20	+	±
Arginine hydrochloride	6.00	±	-
Arginine hydrochloride	26.7	-	-
Arginine	6.0	+	±
Lysine hydrochloride	5.2	+	±
Histidine	4.4	+	±
Shade of bands on SDS-PAGE for high molecular weight polymer products - +++: densely shaded; ++: distinctly shaded; +: shaded, ±: slightly shaded; -: not shaded.			

Example 9: 20k hGH lyophilized preparation

A solution (560 ml) containing 20k hGH (2 mg/ml), creatinine (1.25%, 111 mM) and polysorbate 80 (0.05%), which had been kept frozen, was allowed to thaw in running water and filtered through a 0.22 μm filter. 15 g of D-mannitol and 5.52 g of arginine hydrochloride were added to 510 ml of this filtrate, and dissolved over ice using a stirring bar.

About 14 ml of a 0.5 N sodium hydroxide solution were then added to this solution over ice to adjust the pH to 7.6, after which purified water was added to make the total volume to exactly 600 ml. Using a vial-injection dispenser, 1 ml portions of this solution were dispensed into 2 ml glass vials, which were then lyophilized. For this, the shelf temperature was rapidly decreased from 5°C to -40°C, and preliminary freezing was carried out at -40°C for 5 hours, after which the vials were dried under reduced pressure at a shelf temperature of -25°C for about 50 hours. Next, the shelf temperature was maintained at 15°C under reduced pressure, then the vials were dried for 6 hours to obtain a lyophilized preparation. The resulting cake of the lyophilized preparation had good features, and a clear solution was obtained when reconstituted with injection grade distilled water. The lyophilized preparation was stored at 40°C for 2 months. During storage, the change in appearance of cake and the solution after reconstituted were evaluated, and the amount of related substances was measured. Results are shown in Table 11.

Results confirm that this lyophilized preparation have good appearance, produces no insoluble matter, suppress the production of related substances and contains stable 20k hGH, even after prolonged storage.

Table 11

Stability test of lyophilized preparation			
	Appearance	Appearance of reconstituted solution and related substance (%)	
Day 0	Good	Clear	3.9
After 4 weeks	Good	Clear	5.6
After 8 weeks	Good	Clear	6.2

This invention relates to a stable pharmaceutical preparation containing a human growth hormone having a molecular weight of about 20,000, or a derivative thereof, can be formulated by adding a water soluble heterocyclic compound, such as creatinine, a salt of acetyltryptophane and nicotinamide, to prevent the insolubilization of the human growth hormone or the derivative thereof in an aqueous solution.

In this application, human growth hormone derivatives of human growth hormone having a molecular weight of about 20000 Daltons include compounds functionally equivalent to 20k hGH, homologues of 20k hGH, mono- or polysubstituted 20k hGH, biologicative fragments of 20k hGH, salts of 20k hGH, metabolites of 20k hGH, biologically active analogues of 20k hGH and equivalents thereof.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

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(F) POSTAL CODE (ZIP): 100

(ii) TITLE OF INVENTION: Pharmaceutical Preparation containing Human Growth Hormone

(iii) NUMBER OF SEQUENCES: 2

(iv) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: PatentIn Release #1.0, Version #1.30 (EPO)

(v) CURRENT APPLICATION DATA:
APPLICATION NUMBER: EP 97300607.5

(2) INFORMATION FOR SEQ ID NO: 1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 176 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS:
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(iii) HYPOTHETICAL: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 1:

```
Phe Pro Thr Ile Pro Leu Ser Arg Leu Phe Asp Asn Ala Met Leu Arg
1               5                  10                  15

Ala His Arg Leu His Gln Leu Ala Phe Asp Thr Tyr Gln Glu Phe Asn
            20                  25                  30

Pro Gln Thr Ser Leu Cys Phe Ser Glu Ser Ile Pro Thr Pro Ser Asn
        35                  40                  45

Arg Glu Glu Thr Gln Gln Lys Ser Asn Leu Glu Leu Leu Arg Ile Ser
    50                  55                  60

Leu Leu Leu Ile Gln Ser Trp Leu Glu Pro Val Gln Phe Leu Arg Ser
65                  70                  75                  80
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Val Phe Ala Asn Ser Leu Val Tyr Gly Ala Ser Asp Ser Asn Val Tyr
85 90 95

Asp Leu Leu Lys Asp Leu Glu Glu Gly Ile Gln Thr Leu Met Gly Arg
100 105 110

Leu Glu Asp Gly Ser Pro Arg Thr Gly Gln Ile Phe Lys Gln Thr Tyr
115 120 125

Ser Lys Phe Asp Thr Asn Ser His Asn Asp Asp Ala Leu Leu Lys Asn
130 135 140

Tyr Gly Leu Leu Tyr Cys Phe Arg Lys Asp Met Asp Lys Val Glu Thr
145 150 155 160

Phe Leu Arg Ile Val Gln Cys Arg Ser Val Glu Gly Ser Cys Gly Phe
165 170 175

(2) INFORMATION FOR SEQ ID NO: 2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 176 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS:
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(iii) HYPOTHETICAL: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 2:

Phe Pro Thr Ile Pro Leu Ser Arg Leu Phe Asp Asn Ala Ser Leu Arg
1 5 10 15

Ala His Arg Leu His Gln Leu Ala Phe Asp Thr Tyr Gln Glu Phe Asn
20 25 30

Pro Gln Thr Ser Leu Cys Phe Ser Glu Ser Ile Pro Thr Pro Ser Asn
35 40 45

Arg Glu Glu Thr Gln Gln Lys Ser Asn Leu Glu Leu Leu Arg Ile Ser
50 55 60

Leu Leu Leu Ile Gln Ser Trp Leu Glu Pro Val Gln Phe Leu Arg Ser
65 70 75 80

Val Phe Ala Asn Ser Leu Val Tyr Gly Ala Ser Asp Ser Asn Val Tyr
85 90 95

Asp Leu Leu Lys Asp Leu Glu Glu Gly Ile Gln Thr Leu Met Gly Arg
100 105 110

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Leu Glu Asp Gly Ser Pro Arg Thr Gly Gln Ile Phe Lys Gln Thr Tyr
        115                 120                 125

Ser Lys Phe Asp Thr Asn Ser His Asn Asp Asp Ala Leu Leu Lys Asn
    130                 135                 140

Tyr Gly Leu Leu Tyr Cys Phe Arg Lys Asp Met Asp Lys Val Glu Thr
145                 150                 155                 160

Phe Leu Arg Ile Val Gln Cys Arg Ser Val Glu Gly Ser Cys Gly Phe
                165                 170                 175
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Claims

1. A pharmaceutical preparation containing a human growth hormone having a molecular weight of about 20,000, or a derivative thereof, and a water soluble heterocyclic compound.

2. The pharmaceutical preparation according to claim 1, wherein the water soluble heterocyclic compound is at least one compound selected from the group consisting of creatinine, a salt of acetyltryptophane and nicotinamide.

3. The pharmaceutical preparation according to claim 1 or 2, wherein the pH of an aqueous solution of the preparation is 5 to 8, and optionally the pH of an aqueous solution is controlled by a pH adjusting agent or a buffer solution.

4. The pharmaceutical preparation according to claim 1, 2 or 3, wherein the preparation is a lyophilized preparation containing 1 or 2 kinds of basic amino acids or salts thereof and a nonionic surfactant, and preferably the nonionic surfactant is polysorbate 20 or polysorbate 80, or both.

5. The pharmaceutical preparation according to claim 4, wherein the preparation contains 1 to 20 parts by weight of basic amino acids or salts thereof to 1 part by weight of the human growth hormone having a molecular weight of about 20,000.

6. The pharmaceutical preparation according to any preceding claim, wherein the preparation contains an excipient, and optionally the excipient is a sugar alcohol.

7. A method to prevent insolubilization of a human growth hormone having a molecular weight of about 20,000, or derivative thereof, and to improve stability by adding a water soluble heterocyclic compound to the preparation containing said human growth hormone or derivatives thereof, and preferably the water soluble heterocyclic compound is at least one compound selected from the group consisting of creatinine, salts of acetyltryptophane and nicotinamide, and optionally the pH of an aqueous solution of the preparation is 5 to 8.

8. A method of suppressing production of related substances of a lyophilized preparation containing a human growth hormone having a molecular weight of about 20,000, or a derivative thereof, with time by adding 1 or 2 kinds of basic amino acids or salts thereof and a nonionic surfactant, and optionally the preparation contains an excipient.

9. Human growth hormone having a molecular weight of about 20,000 Daltons or a derivative thereof and functional analogues thereof, for use in therapy.

(19) Europäisches Patentamt
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(54) Pharmaceutical preparation containing human growth hormone

(57) A stable pharmaceutical preparation containing a human growth hormone having a molecular weight of about 20,000, or a derivative thereof, can be formulated by adding a water soluble heterocyclic compound, such as creatinine, a salt of acetyltryptophane and nicotinamide, to prevent the insolubilization of the human growth hormone or the derivative thereof in an aqueous solution.

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EUROPEAN SEARCH REPORT

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EP 97 30 0607

DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	CLASSIFICATION OF THE APPLICATION (Int.Cl.6)
X,D	EP 0 303 746 A (INT MINERALS & CHEM CORP) 22 February 1989 *see in particular claims 1,5; page 3, lines 21-44 *	1,3,6-9	A61K38/27 A61K47/22 C07K14/61
X,D	US 5 496 713 A (HONJO MASARU ET AL) 5 March 1996 *see in particular col. 1, lines 42-47; col. 3, lines 57-67;Example 3 *	9	
A	PATENT ABSTRACTS OF JAPAN vol. 012, no. 312 (C-523), 24 August 1988 & JP 63 084500 A (KUNIO NAKAJIMA), 15 April 1988 * abstract *	1-8	
X		9	
X	WO 92 03478 A (GENENTECH INC) 5 March 1992 * see in particular Table 7 (Example 9)*	9	
A	RASOOL ET AL: "Solubility Enhancement of Some Water-Insoluble Drugs in the Presence of Nicotinamide and Related Compounds" JOURNAL OF PHARMACEUTICAL SCIENCES, vol. 80, no. 4, 1991, pages 387-393, XP002092787 United States	1-8	TECHNICAL FIELDS SEARCHED (Int.Cl.6) A61K
A,D	US 4 980 165 A (ISAACS BENJAMIN S ET AL) 25 December 1990 * see in particular col. 2, lines 5-36; Table V *	1-8	

The present search report has been drawn up for all claims

Place of search	Date of completion of the search	Examiner
MUNICH	10 February 1999	Isert, B

CATEGORY OF CITED DOCUMENTS

X : particularly relevant if taken alone
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E : earlier patent document, but published on, or after the filing date
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L : document cited for other reasons

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This annex lists the patent family members relating to the patent documents cited in the above-mentioned European search report. The members are as contained in the European Patent Office EDP file on. The European Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

10-02-1999

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
EP 0303746 A	22-02-1989	ES 2044941 T	16-01-1994
		HU 44706 A	28-04-1988
US 5496713 A	05-03-1996	DE 69318909 D	09-07-1998
		DE 69318909 T	24-12-1998
		EP 0587427 A	16-03-1994
		JP 6269292 A	27-09-1994
WO 9203478 A	05-03-1992	CA 2087267 A	18-02-1992
		EP 0550629 A	14-07-1993
		JP 6500559 T	20-01-1994
US 4980165 A	25-12-1990	CA 2045562 A	28-07-1990
		DE 69008513 D	01-06-1994
		DE 69008513 T	24-11-1994
		DK 455725 T	05-09-1994
		EP 0455725 A	13-11-1991
		ES 2055902 T	01-09-1994
		JP 2636499 B	30-07-1997
		JP 4503070 T	04-06-1992
		WO 9008557 A	09-08-1990